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Published in:
Molecular Plant-Microbe Interactions

DOI:
10.1094/MPMI.2004.17.7.729

Publication date:
2004

Citation for published version (APA):
Gjetting, T., Carver, T. L. W., Skøt, L., & Lyngkjaer, M. F. (2004). Differential gene expression in individual papilla-resistant and powdery mildew-infected barley epidermal cells. *Molecular Plant-Microbe Interactions*, *17*(7), 729-738. https://doi.org/10.1094/MPMI.2004.17.7.729

MPMI Vol. 17, No. 7, 2004, pp. 729–738. Publication no. M-2004-0507-01R.

Differential Gene Expression in Individual Papilla-Resistant and Powdery Mildew-Infected Barley Epidermal Cells

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Submitted 7 November 2003. Accepted 1 March 2004.

Resistance and susceptibility in barley to the powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*) is determined at the single-cell level. Even in genetically compatible interactions, attacked plant epidermal cells defend themselves against attempted fungal penetration by localized responses leading to papilla deposition and reinforcement of their cell wall. This conveys a race-nonspecific form of resistance. However, this defense is not complete, and a proportion of penetration attempts succeed in infection. The resultant mixture of infected and uninfected leaf cells makes it impossible to relate powdery mildew-induced gene expression in whole leaves or even dissected epidermal tissues to resistance or susceptibility. A method for generating transcript profiles from individual barley epidermal cells was established and proven useful for analyzing resistant and successfully infected cells separately. Contents of single epidermal cells (resistant, infected, and unattacked controls) were collected, and after cDNA synthesis and PCR amplification, the resulting sample was hybridized to dot-blots spotted with genes, including some previously reported to be induced upon pathogen attack. Transcripts of several genes, (e.g., *PR1a*, encoding a pathogenesis related protein, and *GLP4*, encoding a germin-like protein) accumulated specifically in resistant cells, while *GRP94*, encoding a molecular chaperone, accumulated in infected cells. Thus, the single-cell method allows discrimination of transcript profiles from resistant and infected cells. The method will be useful for microarray expression profiling for simultaneous analysis of many genes.

Additional keywords: single-cell analysis

Plant cell responses to attack by pathogenic obligate biotrophic fungi involve rapid and dramatic physiological and metabolic reorganization. Conidia of the barley powdery mildew fungus (*Blumeria graminis* DC Speer f. sp. *hordei* Marchal) germinate and form a mature appressorium with a single apical lobe by around 10 h after inoculation onto host leaves. Starting at approximately 12 h, a penetration peg emerging from beneath the appressorial lobe attempts to penetrate the barley epidermal cell wall. This induces a range of rapid epidermal cell responses, including major cytoskeletal rearrangement, the dynamic aggregation of cytoplasm, and cell nucleus translocation to the site of attempted fungal penetration. Response can result in local production of fungitoxic factors, such as phenolics and reactive oxygen species, and deposition of a papilla that reinforces the plant cell wall at the site of attempted penetration (Zeyen et al. 2002). The speed and degree of host cell response appears to determine the efficiency of this initial defense against attempted penetration. If an effective papilla forms and penetration fails, the pathogen then differentiates a second appressorial lobe at about 18 h, from which it again attempts penetration. Thus, the presence of a second appressorial lobe and of a papilla beneath the first lobe is evidence of failed attack from the first lobe. If penetration succeeds, cells of plants possessing race-specific resistance genes recognize avirulent fungal isolates and this initiates programmed cell death leading to a hypersensitive response (HR). However, if the fungus is virulent, most penetrated cells survive, and the fungus forms an intracellular haustorium by around 15 h. Nutrients absorbed by haustoria support ectophytic hyphal development, the formation of further haustoria in surrounding plant epidermal cells, and sporulation from aerial conidiophores. In a compatible interaction, individual colonies may produce up to 5,000 haustoria and conidiophores and generate 200,000 conidia during their active life (Hirata 1967).

Plant defense responses are primarily controlled by transcriptional activation of specific stress genes and regulation of their temporal and spatial expression (Glazebrook 2001; Rushton and Somssich 1999; Singh et al. 2002). Several of these, members of the so-called pathogenesis-related (PR) genes, are induced in cereals by *B. graminis* attack (Collinge et al. 2002; Van Loon and Van Strien 1999). However, in all cases, *B. graminis* attack induces indistinguishable expression profiles in both resistant and susceptible whole-leaf samples of barley (Gregersen et al. 1997). The reasons for this surprising finding are unknown, but it may be that PR gene induction relates to papilla formation, which occurs in both resistant and susceptible barley. Whether or not this is so, it is well established that epidermal cells of leaves from susceptible and resistant plant genotypes show a mosaic of responses with respect to forming effective papillae or allowing pathogen penetration. Therefore, understanding the involvement of response gene activation in resistance is impossible if analyses use whole-leaf or even isolated total epidermal tissues. These confound outcomes from cells forming effective papillae and cells that are penetrated. Gene expression profiling of individual attacked plant cells offers a means to overcome this problem.

Recent technological advances allow simultaneous measurement of gene expression patterns for tens of thousands of genes through cDNA microarrays, serial analysis of gene expression, and expressed sequence tag (EST) sequencing

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(Quackenbush 2001). The application of gene expression profiling to individual plant cells attacked by pathogens could provide valuable understanding of temporal and spatial gene expression patterns. In the past, such analyses required large quantities of mRNA, limiting profiling studies to examination of extracts from large cell populations, such as provided by bulk cell cultures, entire tissues, or organs. However, over the last decade, the development of reverse transcription-polymerase chain reaction (RT-PCR) amplification procedures has allowed gene expression profiling from small samples of human cells (Dixon et al. 1998; Theilgaard-Mönch et al. 2001) and even from single cells (Alsbo et al. 2001; Chiang 1998; Eberwine et al. 1992; Levsky et al. 2002; Steuerwald et al. 2000; Wang and Stollar 2000). In a few cases, such methods have been applied to small samples of plant cells (Asano et al. 2002; Brandt et al. 1999, 2002; Dresselhaus et al. 1994; Gallagher et al. 2001; Karrer et al. 1995; Richter et al. 1996). A major problem in plants is to sample individual cells. This has been solved by using protoplasts (Dresselhaus et al. 1994; Richter et al. 1996), by microsampling using glass capillaries (Brandt et al. 1999, 2002; Gallagher et al. 2001; Karrer et al. 1995), or laser microdissection (Asano et al. 2002). Dresselhaus and associates (1994) reported the construction of a cDNA library from 128 maize protoplasts, and *GAPDH* gene expression in a single maize protoplast was detected by RT-PCR (Richter et al. 1996). Likewise, detection of specific gene transcripts (peroxidase, rubisco, and starch phosphorylase) was possible by RT-PCR in microsamples from single cells of intact plant tissue (Brandt et al. 1999). A method for amplification of cDNA following single-cell sampling was developed by Karrer and associates (1995), and using this method, they, and later Gallagher and associates (2001), showed that it was possible to detect specific gene transcripts in cDNA pools amplified from specified plant cell types. A similar method was used to construct a cDNA library from 150 microdissected rice phloem cells (Asano et al. 2002). Very recently, large-scale gene expression profiling in extracts from a few specific *Arabidopsis* cells was achieved by combining mRNA amplification and cDNA array hybridization using a modified differential display protocol (Brandt et al. 2002).

This paper presents data on the simultaneous determination of expression levels of various genes in extracts from single epidermal cells of barley attacked by *B. graminis*, including genes encoding for PR proteins (i.e., PR-1, PR-17, chitinases, and taumatin-like protein), phenylpropanoids (i.e., phenylalanine ammonia-lyase and chalcone synthase), structural changes (i.e., oxalate oxidase, oxalate oxidase-like protein, and peroxidases), and regulatory proteins (i.e., 14-3-3 protein and endoplasmin). Previous studies have indicated that the transcription of several of these genes is up-regulated in response to *B. graminis* attack. However, the role of most of these genes in disease resistance was unclear because they were extracted from entire leaves or total epidermis containing a mix of cells that were successfully penetrated and cells that contained effective papillae. Our approach overcame this problem by using mRNA extracted from individual barley epidermal cells that microscopy showed to have resisted penetration having formed effective papillae or to have been penetrated successfully so that they contained a fungal haustorium. We show that it was possible to identify genes whose transcripts were apparently unaffected by the outcome of attack and others that were up-regulated specifically or preferentially in resistant or penetrated cells.

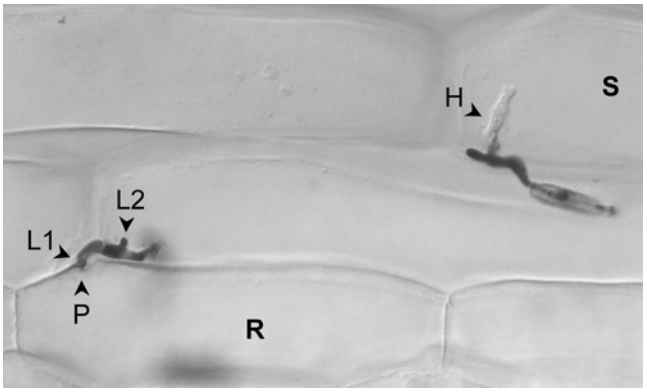

Fig. 1. Different outcomes of attempted penetration by *Blumeria graminis* into leaf epidermal cells of the susceptible barley line Pallas, 18 h after inoculation. One of the barley epidermal cells has resisted penetration (**R**) from the first *B. graminis* appressorial lobe (*L1*). This is recognized by the presence of a subtending papilla (*P*) and by the fact that a second *B. graminis* appressorial lobe (*L2*) has differentiated. One cell has been penetrated successfully (**S**) and contains a rudimentary haustorium (*H*) beneath the first appressorial lobe.

RESULTS

Sampling.

Individual epidermal cells of Pallas barley that had resisted penetration by *B. graminis* and cells that had been penetrated were easily distinguished by microscopy of living plant leaves 18 h after inoculation (Fig. 1). Resistant cells were recognized by the presence of a papilla subtending the first lobe of appressoria that had subsequently formed a second lobe, while penetrated cells contained a rudimentary haustorium. The contents of five individual resistant cells, five penetrated cells, or five uninoculated control cells, were collected in microcapillaries as single samples and were delivered into extraction buffer within 90 s of commencing collection.

Generation and analysis of amplified cDNA pools from single epidermal cells.

When mRNA present in the single-cell samples was purified, used for cDNA synthesis, and amplified by PCR, a smear was obtained following agarose gel electrophoresis. In all cases, maximal intensity was obtained between 400 and 800 bp, irrespective of whether epidermal cells were of the resistant or penetrated class or were uninoculated controls. This smear presumably indicates that PCR products contained a population of both full-length and truncated transcripts. However, the smears produced from the different classes had very consistent characteristics, as illustrated in Figure 2A, which shows the consistency between four examples of amplified cDNA pools from uninoculated control cell samples collected from different leaves at different times.

The integrity of amplified cDNA pools was tested by PCR, using primers for known, specific genes. Ubiquitin is expressed in all plant cells (Herschko and Ciechanover 1998), and the results of amplification of the barley ubiquitin-conjugating enzyme gene *UBC* are shown in Figure 2B. From this, it is clear that *UBC* cDNA was present in single-cell cDNA samples from all cell classes as well as in cDNA prepared from whole-plant total RNA. Similar results were obtained with α-tubulin 2 (*αTUB2*) (not shown). These findings indicated the potential for using cell-specific amplified cDNA pools to generate transcription profiles relating directly to different outcomes of the barley-powdery mildew interaction. However, a major concern when analyzing transcript profiles from small cell samples is that PCR-mediated amplification of cDNA can fail to maintain the ratios of gene transcripts present in the original sample (Hertzberg et al. 2001), due to

small and random differences in amplification efficiency between individual templates in the cDNA population, also termed the Monte Carlo effect (Karrer et al. 1995). We found this to be the case. Thus, amplified cDNA pools prepared from five-cell samples varied significantly between independent experiments in transcript profiles of different genes (data not shown). Bulking several independent cDNA pools from the same epidermal cell class after PCR amplification reduced variation but failed to provide consistent results (data not shown). However, variation was greatly reduced by increasing the initial quantity of mRNA in cell extracts. This was done by combining four microsamples (giving a total of 20 cell extracts) before PCR amplification.

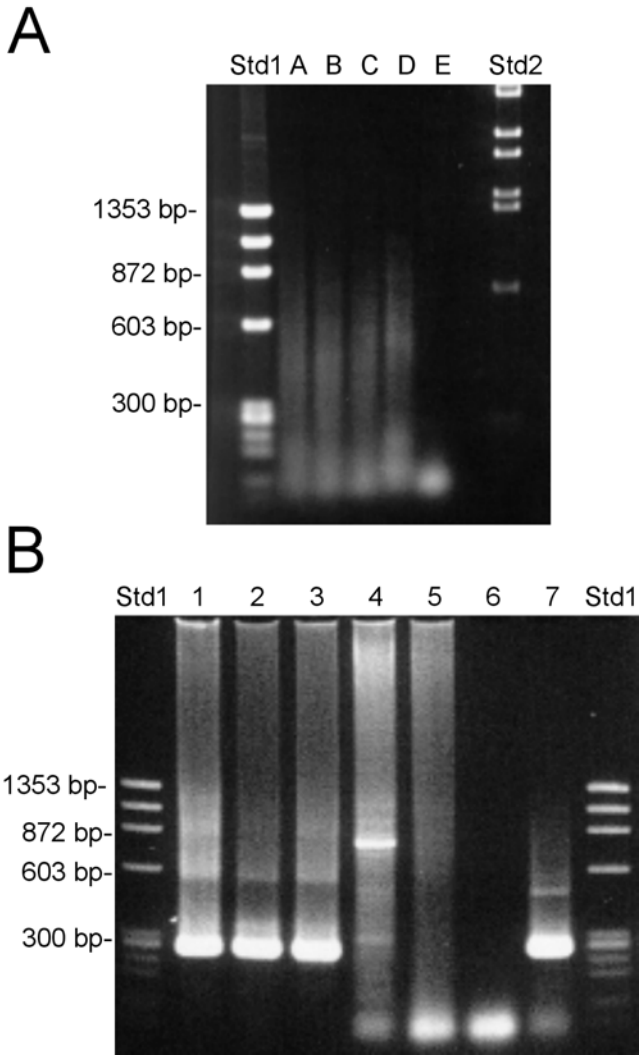

Fig. 2. Poymerase chain reaction (PCR)-amplified single-cell cDNA pools and ubiquitin-conjugating enzyme e2 (*UBC*)-specific PCR. **A,** Single-cell cDNA pool amplified by universal primers annealing to the poly(dA)-tail and the 5′-added linker, analyzed by agarose gel electrophoresis. Lanes A through D represent samples produced from uninoculated control barley epidermal cells. Lane E is a blank control. Std1 = PhiX174/*Hae*III-digest. Std2 = Lambda/*Bst*EII-digest. **B,** *UBC*-specific PCR using different templates. Lane 1, Amplified single-cell cDNA pool prepared from resistant epidermal cells that contained a papilla. Lane 2, Amplified single-cell cDNA pool prepared from epidermal cells that were successfully penetrated and contained a fungal haustorium. Lane 3, Amplified single-cell cDNA pool prepared from uninoculated control cells. Lane 4, Bead control processed in the same way as samples. Lane 5, Blank control from the amplified cDNA pool. Lane 6, Blank control. Lane 7, Positive control (cDNA from whole-leaf material from Pallas line seedlings).

Figure 3 shows transcript profiles of 19 barley genes in microsamples from uninoculated cells either bulked after PCR amplification or combined before PCR amplification and subjected to different numbers of thermal cycles. The dot-blots were spotted with 19 barley cDNA fragments. The dot-blots were hybridized with samples prepared from either a bulk of five amplified cDNA pools (each prepared from five cells) combined after 35 cycles of PCR (I) or from four microsamples (twenty cells in total) combined before 25 (II), 35 (III), and 45 (IV) cycles of PCR amplification. When radioactive sample materials were derived from samples combined before PCR amplification (Fig. 3, dot-blots II through IV), hybridization intensity varied greatly according to the number of PCR cycles used. However, despite the fact that the blots were obtained from different, independent experiments, the transcript profiles were extremely consistent between blots, as indicated by internal ratios between dot intensities within filters. Although the transcript profile obtained from bulking cDNA samples after amplification (Fig. 3, dot-blot I) resembles the profiles from the combined samples, it was evident that transcript profiles of the five individual samples within the bulk differed greatly from filters II through IV (data not shown). Thus, although bulking cDNA samples after amplification gave unreliable data, combining four samples prior to PCR amplification provided reproducible results.

Gene expression profiles
from healthy and *B. graminis*-attacked barley.

Dot-blot filters were prepared using 20 barley genes, including a number thought to be involved in defense-related activity, such as PR protein synthesis, H_2O_2 generation, phenylpropanoid biosynthesis, and regulatory processes, as well as five 'housekeeping' genes (Table 1). The filters were used to obtain transcript profiles in whole-leaf extracts from healthy and *B. graminis*-inoculated plants and in extracts from 20 single epidermal cells combined prior to PCR amplification (as described above). These single-cell extracts were from resistant epidermal cells having formed effective papillae, penetrated cells containing a haustorium, and uninoculated control cells. Spot intensities were quantified by phosphorimaging. Although no robust measure for internal normalization of samples was available or predicted in advance, we repeatedly found that *UBC* transcript was detected at similar levels in all single-cell samples, indicating that it was applicable for internal stan-

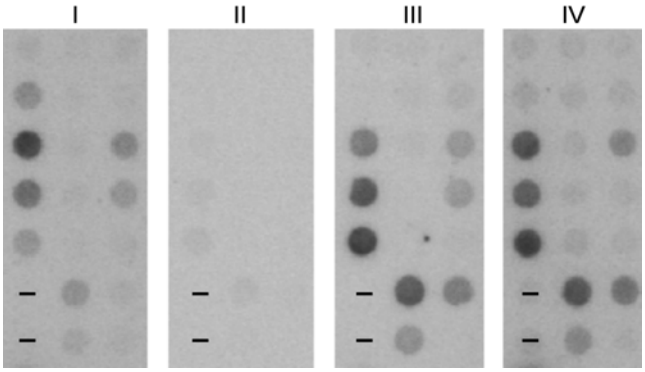

Fig. 3. Dot-blots comparing transcript intensities in which microsamples were either bulked after polymerase chain reaction (PCR) amplification or combined before 25, 35, or 45 cycles of PCR amplification. Four identical filters (I, II, III, and IV) spotted with 19 cDNA fragments (three housekeeping genes, eight defense genes, and eight barley expressed sequence tags) hybridized with five amplified cDNA pools (each prepared from five cells) bulked after 35 cycles of PCR (I) or four samples (twenty cells in total) that were combined before 25 (II), 35 (III), or 45 (IV) cycles of PCR amplification. (The short bold lines indicate empty wells).

dardization. Indeed, statistical analysis of variance (data not shown) applied to data from all repetitions of all experiments identified *UBC* as the most constant of the analyzed transcripts. Furthermore, a Northern analysis of the *UBC* transcript in Pallas whole-leaf RNA showed that it was equally expressed during the first 24 h after powdery mildew attack (T. Gjetting, *unpublished data*). Hence, *UBC* was used for internal standardization.

Whole-leaf cDNA samples.

Whole-leaf cDNA prepared from Pallas (susceptible) and P22 (penetration resistant) plants 18 h after *B. graminis* inoculation and from uninoculated Pallas plants gave expression profiles (Fig. 4A, left panel) that were as expected from previous studies (Christensen et al. 2002; Gregersen et al. 1997; Schweizer et al. 1999a; Walther-Larsen et al. 1993; Wei et al. 1998; Zhou et al. 1998). The profile from uninoculated Pallas plants showed that most of the genes included on the dot-blot, though detectable, showed weak hybridization intensities, indicating low levels of gene expression. As expected however, there was marked intensification of hybridization in inoculated Pallas plants, indicating strongly up-regulated transcription, for the response genes *Prx8*, *GLP4*, *PR17a*, *PR17b*, *PR1a*, *PR1b*, *PR3a*, *PR3b*, and *PR5b*. Elevated, but moderate hybridization signal was evident for *Prx7*, *OxOa*, *GRP94*, *14-3-3a*, and phenylalanine ammonia

Table 1. cDNA fragments spotted on the dot-blot filters

	Lane I			Lane II		
Row	**Name[a]**	**Presumed protein function**	**GenBank accession no.**	**Name**	**Presumed protein function**	**GenBank accession no.**
1	Prx7*	Peroxidase	X62438	PR3a*	Chitinase	X78671
2	*Prx8**	Peroxidase	X58396	*PR3b**	Chitinase	X78672
3	*OxOa**	Oxalate oxidase	Y14203	*PR5b**	PR-5 protein	AJ001268
4	*GLP4**[b]	Oxalate oxidase-like protein	X93171	*CHS2**	Chalcone synthas	Y09233
5	*GRP94**	Endoplasmin	X67960	*PAL**	Phenylalanine ammonia-lyase	Z49146
6	*14-3-3a**	14-3-3 protein	X62388	*EF1α*[δ]	Elongation factor 1α	Hv.3305
7	*PR17a**	Hypothetical protein	Y14201	*VAG1*[δ]	Vacuolar ATP synthase subunit G	Hv.3786
8	*PR17b**	Hypothetical protein	Y14202	*UBC*[δ]	Ubiquitin-conjugating enzyme e	Hv.4038
9	*PR1a**	PR-1a protein	X74939	*αTUB2*[δ]	Alpha-tubulin 2	Y08490
10	*PR1b**	PR-1b protein	X74940	*ACT*[δ]	Actin	U21907

[a] * indicates a defense-related barley gene and δ indicates a 'housekeeping' gene.
[b] *GLP4* was formerly referred to as *OxOLP* but was recently placed into sub-family 4 of the germin-like protein (GLP) superfamily (Druka et al. 2002).

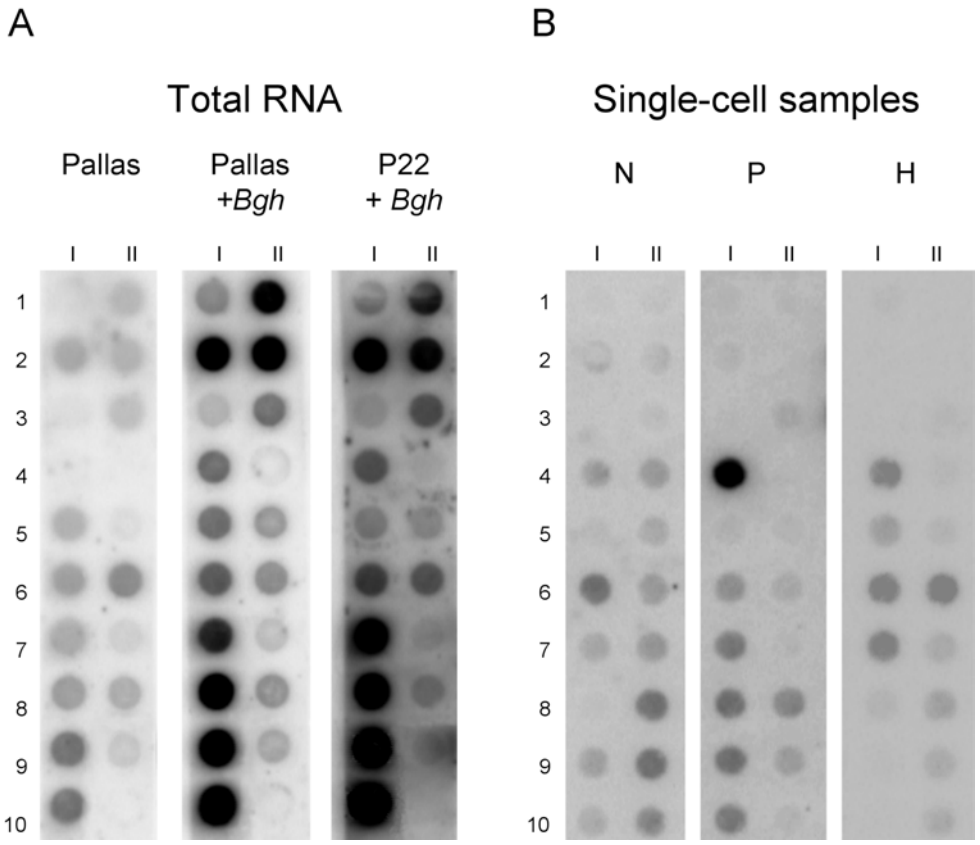

Fig. 4. Dot-blots of selected genes showing comparisons of transcript profiles in cDNA prepared from whole leaves and cDNA amplified from single-cell samples. Six identical membranes dotted with cDNA fragments from 20 barley genes. These include 15 genes known to be up-regulated in barley attacked by powdery mildew (lane I, rows 1 to 10 and lane II, rows 1 to 5) and five 'housekeeping' genes (lane II, rows 6 to 10). **A,** Labeled samples made from barley whole-leaf cDNA prepared from uninoculated Pallas leaves and from Pallas (susceptible) or P22 (penetration resistant) plants 18 h after *Blumeria graminis* inoculation. **B,** Three cDNA samples prepared from 20 Pallas epidermal cells that were either uninoculated controls (N) or were sampled 18 h after *B. graminis* attack and were resistant, papilla-containing cells (P) or cells that were penetrated and contained a haustorium (H).

lyase (*PAL*). Expression of chalcone synthase (*CHS2*) was not stimulated, confirming that this gene is not induced during the early stages of *B. graminis* infection (Christensen et al. 1998; Gregersen et al. 1997). In contrast to the response genes, the housekeeping genes elongation factor 1-α (*EF1α*), vacuolar ATP synthase subunit G 1 (*VAG1*), *UBC*, *αTUB2*, and actin (*ACT*) were more or less unaffected by *B. graminis* attack. Interestingly, no obvious difference in expression profiles was evident between the susceptible Pallas and the penetration-resistant P22 barley lines after *B. graminis* attack.

Single-cell cDNA samples.

Expression profiles of cDNA prepared using single-cell extracts from the Pallas line are shown in Figure 4B, and the quantification of mean spot intensities derived from three individual experiments are shown in Figure 5. The expression profile from uninoculated control cells (Fig. 4B, left panel) showed low, weak, or undetectable levels of expression for all genes, and in this way, it resembled the profile from whole-leaf extracts of uninoculated Pallas. However, although transcript levels of some genes remained unchanged in attacked cells, some were specifically up-regulated according to the outcome of attempted penetration. Only one gene, *αTUB2*, was down-regulated in both resistant cells forming effective papillae and infected, haustorium-containing cells.

In single-cell samples from resistant epidermal cells that formed effective papillae (Fig. 4B, middle panel), the gene *GLP4*, which codes for an epidermis-specific oxalate oxidase-like protein (Wei et al. 1998), was very strongly up-regulated. As in inoculated whole-leaf extracts, the genes of unknown function *PR17a*, *PR17b*, *PR1a*, and *PR1b* were also clearly induced. In contrast to whole leaves, the genes *Prx8*, *PR3a*, and *PR3b*, known to be expressed in mesophyll tissue (Gregersen et al. 1997; Wei et al. 1998), were not induced, indicating that the extracts from epidermal cells were free of contamination by mesophyllic material.

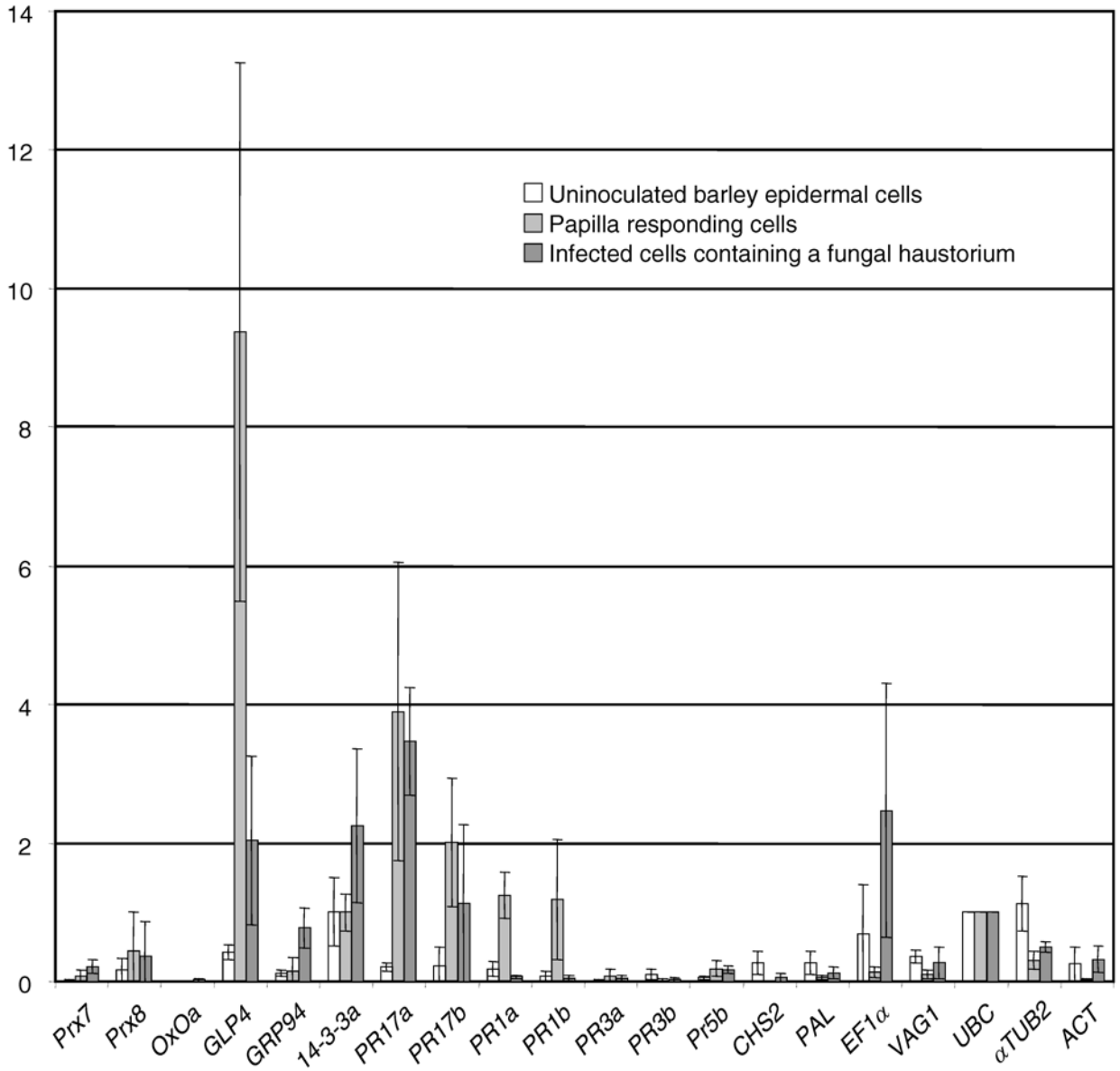

Fig. 5. Quantified expression-levels of the selected genes in amplified single-cell cDNA pools from control cells, resistant papilla-containing cells, and penetrated cells containing a *Blumeria graminis* haustorium, relative to ubiquitin-conjugating enzyme e2 (*UBC*) expression in the same cell class. Quantification was by measuring spot intensities on dot-blots derived from three individual, replicate experiments, correcting for background and standardization against the equivalent *UBC* signal intensity. Data show the mean standardized intensities for transcripts of each gene expressed in each cell class. Error bars indicate standard deviations.

There was no detectable induction of *PR1a* and *PR1b* in extracts from haustorium-containing epidermal cells, although *PR17a* and *PR17b* were induced almost to the same degree as in cells containing a papilla (Fig. 4B, right panel). Compared with uninoculated control cells, *GLP4* was induced but to a much lower level than in papilla-containing cells. By contrast, *GRP94* was clearly and specifically up-regulated in haustorium-containing cells, showing no induction in cells containing a papilla. The same tendency was shown by *14-3-3a* and *EF1α*, although for these transcripts, differences with controls were not statistically significant.

DISCUSSION

An important aim of the present study was to establish whether transcript analysis based on RT-PCR applied to extracts from small numbers of barley epidermal cells can be used to discriminate between 'defense genes' involved in cellular resistance to powdery mildew attack and genes involved in the major physiological and metabolic reorganization of host cellular activity (Green et al. 2002) resulting from successful infection by a virulent fungal isolate. To date, using analyses based on whole organs or tissues, it has been difficult to do this because of the mixed responses seen even in susceptible host leaves, whereby some epidermal cells are successfully infected, others form effective defensive papillae, and some die as a result of attack (Lyngkjaer and Carver 1999). Data from such analyses are therefore interpreted by correlative evidence and by 'best guessing' from evidence of gene function. This problem was recently highlighted in a parallel study by Mould and associates (2003), which focused on discriminating between events that precede either establishment of successful infection or HR in the cowpea-cowpea rust system. By using RT-PCR on extracts from attacked single cells of a susceptible line and a line with resistance-conditioning HR, they identified genes that were expressed commonly or specifically in cells of the susceptible or resistant line. Their studies illustrate the power of the single-cell analysis approach for studies of plant pathogenesis. However, the cowpea rust system differs from the cereal powdery mildew and many other plant pathogen systems in that cowpea cells show no nonspecific defensive responses to penetration. In cereals, papilla deposition is a key defense against powdery mildew attack (Zeyen et al. 2002), and hence, understanding the cellular basis of this nonspecific response is of great importance. Our results show that this, too, will be facilitated by single-cell transcript analysis.

For the present investigation, we selected various genes on evidence of their up-regulation in response to *B. graminis* attack or their putative function (Christensen et al. 1998, 2002; Gregersen et al. 1997; Thordal-Christensen et al. 1992; Wei et al. 1998). As expected (Clark et al. 1994; Gregersen et al. 1997), although B. graminis attack clearly induced some of these genes in whole-leaf samples, no obvious differences in expression profiles were evident between the susceptible Pallas barley line and the isoline P22 (carrying the *mlo5* allele), which shows almost total, papilla-based penetration resistance. This failure to discriminate, even between genotypes showing dramatically different phenotypes, reflects others' findings from whole-leaf analyses (Gregersen et al. 1997; Wei et al. 1998) and is probably explained by the mix of successful and failed penetration attempts resulting from attack on leaf epidermal cells of even nominally 'susceptible' plants. By contrast, from single-cell analyses, the transcript profiles for some genes differed clearly between resistant cells that formed effective papillae and infected, haustorium-containing cells, even though these cells came from the same plant genotype.

The genes *VAG1* (vacuolar ATPase G1), *ACT* (actin), and *UBC* (ubiquitin-conjugating enzyme e2) showed indistinguishable and low levels of expression in control cells and in papilla- and haustorium-containing cells. These genes were therefore candidates to provide internal standards against which other transcripts could be evaluated. Since *UBC* gave the most consistent results, it was selected as the standard.

Of the PR genes investigated, only *PR1a* and *PR1b* were induced specifically in resistant cells forming effective papillae. These genes encode proteins of unknown function, and our results indicate the need for further investigation of their roles. By contrast, *PR17a* and *PR17b* were induced in both resistant cells forming effective papillae and infected, haustorium-containing cells, and our data, taken from a single timepoint (18 h after inoculation), do not indicate whether or not they influence penetration resistance. Since the genes *PR3a*, *PR3b*, and *PR5b* were not induced in attacked epidermal cells (confirming the findings of Gregersen and associates [1997]), their relevance to penetration resistance in the barley mildew system is also questionable, although as they were all strongly induced in whole-leaf extracts, it is possible that their expression in tissues underlying the epidermis may influence epidermal cell responses indirectly. The same is true of *OxOa.*

Three genes, *Prx7, Prx8*, and *GLP4*, are involved in the production or removal of reactive oxygen species (Collinge et al. 2002) generated as an inevitable result of cellular metabolism that is necessarily increased by cytological responses of plant cells under attack by *B. graminis*. Transcripts of *Prx7* and *Prx8* were present at low levels in uninoculated epidermal cells, and their abundance did not increase after attack. Therefore, we have no evidence to confirm their roles in resistance, although other data suggests that the peroxidase encoded by *Prx8* contributes to penetration resistance through local wall strengthening (Schweizer et al. 1999b) and *Prx7* has been implicated in dimerization of antifungal hordatines (Kristensen et al. 1999). However, in accordance with previous data (Gregersen et al. 1997; Kristensen et al. 1999), *GLP4* transcript accumulated strongly in attacked epidermal cells, and our single-cell analysis revealed that its accumulation was significantly greater in cells containing effective papillae than in infected cells. *GLP4* encodes for an oxalate oxidase-like protein (formerly named *HVOxOLP*; Wei et al. 1998) that was recently placed into subfamily 4 of the germin-like protein superfamily (Druka et al. 2002), and the protein is thought to be responsible for generation of H_2O_2 in papillae (Christensen et al. 2004; Hüeckelhoven et al. 2001; Zhang et al. 1995). Our data are compatible with this hypothesis. The more modest elevation of *GLP4* transcripts in cells containing haustoria may reflect oxidative activity resulting from haustorial formation or feeding activity or from the production and construction of the haustorial neck collar, which contains some of the components present in papillae.

The genes *CHS2* and *PAL* code for enzymes involved in phenylpropanoid biosynthesis. It was not surprising that only low levels of *CHS2* transcript were detected in control and attacked epidermal cells, because previous studies showed no accumulation until 4 to 6 days after inoculation (Gregersen et al. 1997), far later than our extracts were collected. However, the absence of *PAL* transcript from resistant cells was surprising. This is because PAL enzyme activity appears essential for effective papilla formation in barley, since inhibition of the enzyme (Carver et al. 1994), even in monolayers of living barley epidermal cells (Zeyen et al. 1995), dramatically reduces the effectiveness of papilla defense, increasing the frequency of penetration and haustorium formation. However, there are at least six different *PAL* genes in barley (Kervinen et al. 1997), and it is possible that the one used in our dot-blots is meso-

phyll-specific. Thus, it may not have hybridized with the form amplified from resistant cells containing effective papillae but only with one or more forms present in whole-leaf extracts. Further studies are needed to test this possibility. Alternatively, the temporal precision of our sample extraction in relation to development of the host pathogen interaction may explain our failure to detect *PAL* transcript in resistant cells. Even where mRNA is extracted from whole leaves under attack by a population of fungal germlings developing relatively asynchronously (so that various stages of pathogen attack and consequent host response are involved in the sample), peak *PAL* transcript accumulation is seen at around 15 h after inoculation, and it declines rapidly thereafter (Clark et al. 1994). Our extracts were taken at a single timepoint 3 h later than this peak. Furthermore, we extracted from cells in which papilla deposition had effectively blocked penetration from the first appressorial lobe, as shown by differentiation of a second appressorial lobe. Thus, it is possible that by the time of extraction, the need for PAL activity had passed and *PAL* transcription had declined to control levels. If true, this emphasizes the need for extreme care in interpreting data arising from sampling at a single timepoint and that timecourse studies are essential for complete understanding. Following this argument, the absence of increased transcription for a particular gene (such as we found with certain PR genes and *Prx7* and *Prx8*) at a particular sample time cannot be taken as evidence for its lack of involvement in the temporally dynamic processes of papilla deposition.

The finding that transcripts of certain genes accumulated in haustorium-containing cells suggests their involvement either in supplying nutrient to the parasite or in cytological modifications made to accommodate the developing haustorium, e.g., formation of the extrahaustorial membrane that envelops the haustorium. These cells showed a marked increase in transcription for *GRP94,* which was previously shown to be induced in barley by both heat shock and powdery mildew attack (Walther-Larsen et al. 1993). Furthermore, in whole-leaf samples, *GRP94* accumulated in plants from the Pallas line (in which many penetration attempts succeed and haustoria form) but not in those from the P22 barley line in which penetration is prevented. The gene encodes a 94-kDa glucose-regulated protein (GRP94), endoplasmin, belonging to a class of HSP90 proteins associated with endoplasmic reticulum (ER) (Csermely et al. 1998). Although ER undergoes major reorganization within infected cells, forming a dense network around developing haustoria (Green et al. 2002), the functions of GRP94 in plant pathogenesis are unknown. Nevertheless, in mammalian cells, its very close homologues act as molecular chaperones in the folding of ER-expressed proteins destined for plasma membrane localization or secretion (Csermely et al. 1998), and they are strongly induced in stressed cells and tumor cells, being expressed on the cell membrane surface, with a possible role in antigen presentation and tumor rejection (Srivastava et al. 1986). These proteins may also be involved in preventing apoptosis due, for example, to oxidative stress (Punyiczki and Fésüs 1998). It is plausible that barley GRP94 could have similar roles in preventing death of infected cells or in the intimate relationship between plant cell ER and the extrahaustorial membrane that is thought to be derived from an invagination of the host plasma-membrane and through which all nutrients must pass before take up by the fungus (Green et al. 2002), or both. Recently, it was reported that a plant HSP90 protein interacts directly with the host defense *R* gene-dependent resistance modulator RAR1 and its interactor SGT1 (Shirasu and Schulze-Lefert 2003), suggesting a role for chaperones in disease resistance. Further single-cell analyses of *GRP94* transcript expression in barley powdery mildew interactions are in progress toward understanding the roles of this apparently important gene.

Of the two other genes showing increased transcription in haustorium-containing cells, *14-3-3a* encodes a protein involved in regulation of transport across plant plasma membranes (Finnie et al. 2002), and it seems reasonable that it is required to support increased transport activity arising from parasitism. Although *EF1α* was expressed relatively strongly in haustorium-containing cells and it was barely detectable in resistant cells containing effective papillae, the data were highly variable between experiments, and we cannot interpret their biological significance.

Only one of the genes we considered, *αTUB2*, coding for α-tubulin 2, was down-regulated in both resistant cells forming effective papillae and infected, haustorium-containing cells. Interestingly, Mould and associates (2003) also found down-regulation of tubulin gene expression in susceptible cowpea epidermal cells attacked by cowpea rust. They thought that, since cells of this plant form no defensive papillae, reduced expression of tubulin genes in susceptible cells may relate to the rapid, localized reduction in microtubules seen at sites of successful fungal penetration. This may also explain our result from haustorium-containing cells. However, it does not explain why we found down-regulation of *αTUB2* in resistant barley cells in which reorganization of microtubules to a focus beneath the attack site is correlated to the formation of effective papillae (Kobayashi et al. 1992, 1997). It may be that either *αTUB2* is not involved in papilla formation or its activity is, in this context, independent of de novo transcription. Alternatively, as suggested for *PAL*, it may be that, by sampling only after papillae had been formed, we missed a phase of up-regulated *αTUB2* transcription that was down-regulated by the time of sampling.

The single-cell analysis approach we have begun to exploit clearly offers a means of elucidating the genetic basis of resistance and susceptibility by avoiding the confounding effects of mixed cellular reactions implicit in whole leaf extracts. It may also help to explain the basis of these mixed cellular reactions. Do some cells resist attack because they are more physiologically competent to respond? Do some cells succumb to infection because the pathogen is, for some reason, more competent to suppress host cell defenses? At present, we can only speculate. Our tests indicate that, by pooling the contents of 20 individual cells and using precise PCR conditions, dependable and reproducible results can be obtained. Further refinements may increase the quality and efficiency of the procedure, but the approach is obviously very powerful. Most importantly, the method is directly up-scalable to DNA microarray or chip hybridization, which will prove invaluable for studying genome-wide transcriptional changes in relation to both successful defense against pathogen attack and the consequences of established parasitism. However, as our experience suggests, data must be interpreted with care, and regard must be paid to the temporal dynamics of plant transcript accumulation.

MATERIALS AND METHODS

Plants, fungi, inoculation, and incubation.

The susceptible barley (*Hordeum vulgare* L.) line Pallas and the near-isogenic *mlo5*-resistant barley line P22 (Kølster et al. 1986) were used for whole-leaf analyses. Pallas seedlings were used for single-cell analyses. Plants were grown to full expansion of the first-formed leaves (9-day-old) under standard conditions of 20 ± 2°C and 250 μmol m^{-2} s^{-1} photon flux density during an 18-h light period. An isolate (A6) of *B. graminis* f. sp. *hordei*, virulent in Pallas but arrested at the penetration stage in P22, was maintained on Pallas seedlings.

Leaves with heavily sporulating colonies were shaken 1 day before inoculation to remove aging conidia and ensure a supply of young conidia for inoculation. The adaxial surface of healthy first-formed leaves was inoculated halfway through the light period, using a settling tower to give 50 or 100 conidia per square millimeter for single-cell or whole-leaf analyses, respectively. Plants were then incubated for 18 h under the standard conditions until extracts were prepared.

Chemicals.

Unless otherwise indicated, chemicals were SigmaUltra grade from Sigma-Aldrich Corp. (St. Louis), enzymes were from Promega (Madison, WI, U.S.A.), and deoxyoligonucleotides were from MWG-Biotech AG (Ebersberg, Germany).

RNA extraction from whole leaves.

Total RNA was extracted from healthy Pallas seedling leaves and from leaves from *B. graminis*-inoculated Pallas and P22 seedlings by grinding in liquid nitrogen, followed by suspension in GTC extraction buffer (guanidine thiocyanate [5 M] Tris-HCl [50 mM, pH 7.5], EDTA [10 mM, pH 8.0], β-mercapto ethanol [8 %]) and centrifugation. The supernatant was filtered, LiCl was added to a final concentration of 3.3 M, and RNA was precipitated at 4°C overnight. Pellet material was repeatedly extracted with acidic phenol/chloroform, followed by reprecipitation from ethanol. RNA integrity was evaluated according to Sambrook and associates (1988) and by RT-PCR testing.

Single-cell analysis.

After inoculation (18 h), living Pallas leaf segments were examined by brightfield microscopy, using a long working distance 20× objective without a coverslip. Two distinct outcomes of the host-pathogen interaction were apparent. Failed penetration from the first appressorial lobe was recognized by the presence of a subtending papilla and by the fact that a second appressorial lobe had differentiated. Successful pathogen penetration was recognized by the presence of a rudimentary haustorium within an epidermal cell directly beneath the first appressorial lobe. As targets for cell content extraction, only type 'B' cells (short epidermal cells having no contact with stomatal complexes) (Koga et al. 1990) in contact with a single appressorium were accepted and only if no other conidia or their germ tubes were present within two cells distance. Dead (collapsed) epidermal cells were avoided.

Extraction from single epidermal cells was performed with an InjectMan NI 2 (Eppendorf AG, Hamburg, Germany) micromanipulator. Microcapillary tubes (5-µm tip diameter) were pulled (P-97 pipette puller; Sutter Instrument Company, San Francisco) from siliconized (Sigmacote) borosilicate capillaries (outer diameter, 1.00 mm; inner diameter, 0.50 mm, length 10 cm) (Sutter Instrument Company). The contents of target epidermal cells punctured by the microcapillary entered it by capillary force. Separately for each interaction outcome (resistant cells containing effective papilla and infected, haustorium-containing cells) and avoiding contact with fungal structures, the contents of five individual Pallas epidermal cells were collected as one sample in the same microcapillary. Samples were then injected (FemtoJet, Eppendorf AG) into a drop of extraction buffer and were mounted on parafilm next to the leaf segment within 90 s. of collection from the first cell. In the same way, uninoculated Pallas epidermal cells were sampled as controls. Four independent samples were prepared for each cell class (resistant, haustorium-containing, and controls). The experiment was replicated three times.

mRNA purification and cDNA synthesis.

The mRNAs in samples were immediately captured and purified on poly(dT) oligonucleotides coupled to magnetic beads (20 µl of resuspended beads per sample) using the Dynabeads mRNA DIRECT micro kit (Dynal, Oslo, Norway), according to the manufacturer's instructions. A magnetic particle concentrator (Dynal) facilitated the change of buffers and reaction conditions between each step of the procedure.

First-strand cDNA synthesis on the magnetic beads was initiated within 8 min, in a total volume of 15 µl containing MMLV (-H) reverse transcriptase (200 U in 1× of the supplied buffer), dNTP (0.5 mM each), and RNasin (20 U) and was incubated at 42°C for 90 min. After cooling to 16°C, a second-strand synthesis mix (140 µl total volume), containing Tris (21 mM), KCl (103 mM), $MgCl_2$ (5 mM), $(NH_4)_2SO_4$ (11 mM), dithiothreitol (4 mM), dNTP (0.2 mM each), NAD^+ (170 µM), *E. coli* DNA ligase (10 U; New England Biolabs, Beverly, MA, U.S.A.), DNA polymerase I (10 U), and RNase H (1 U), was added, and the sample was incubated for 120 min at 16°C. The reaction was continued for another 10 min, after adding 3 U of T4 DNA polymerase to ensure blunt ends of the cDNA. Each sample was purified and then suspended in 40 µl of TE-t (Tris [10 mM, pH 8.0], EDTA [1 mM], Tween-20 [0.1 %]).

Samples (arising from contents of five cells) were either kept separate or four samples from the same cell class (contents of 20 cells in total) were combined before enzyme inactivation (75°C, 15 min). After washing in ligation buffer, a linker (50 nM), prepared from the oligodeoxynucleotides Linker-T7-for (AAT ACG ACT CAC TAT AG) and Linker-T7-rev (P-CTA TAG TGA GTC GTA), was added to the cDNA, followed by overnight incubation at 16°C in 60 µl of 2,000 U T4 DNA ligase (New England Biolabs).

PCR amplification of single-cell cDNA pool.

After washing in Advantage PCR buffer, a volume of 50 µl containing Advantage PCR buffer, dNTP (320 µM), primers (CAT-T7-for [CAT CAT CAT CAT AAT ACG ACT CAC TAT AG] and CTA-T_{15}-rev [CTA CTA CTA CTA TTT TTT TTT TTT TTT], 800 nM each), and Advantage 2 Polymerase Mix (BD Biosciences-Clontech, San Diego, CA, U.S.A.) was used to resuspend beads, and the suspension was transferred to a 0.2-ml PCR tube. PCR was performed as follows: 160 s at 94°C, then 35 cycles of 20 s at 94°C, 20 s at 50°C, 120 s at 72°C, followed by 5 min of incubation at 72°C. Following amplification, 2-µl samples were analyzed by agarose gel (1.5%) electrophoresis.

Gene transcript analysis by PCR.

Amplification by PCR of a 260-bp fragment of the cDNA coding for ubiquitin-conjugating enzyme e2 (*UBC*), using the primers hv_ubiqe2-372-for (GGT TCT GCT TTC AAT CTG CTC GCT G) and hv_ubiqe2-607-rev (GGG AGA CAC ACG CAA CCG ACA AGT A), was performed in 15 µl, using 1 µl of single-cell amplified cDNA pool as template, under the following conditions: 160 s at 94°C, then 35 cycles of 20 s at 94°C, 20 s at 59°C, 20 s at 72°C, followed by 5 min of incubation at 72°C.

Dot-blot hybridization.

A dot-blot with 20 barley cDNA fragments was produced. We selected 14 genes known from previous studies (Gregersen et al. 1997; Thordal-Christensen et al. 2000) to be up-regulated after mildew attack (Table 1) to test induction in our samples. A number of additional genes were used. *PAL* was used because it regulates the biosynthesis of phenolic compounds involved in penetration resistance (Clark et al. 1994). *EF1α*, *VAG1*, and *UBC* were used because they are highly expressed in EST sequencing of barley epidermal tissue (Unigene system; National

Center for Biotechnology Information, Bethesda, MD, U.S.A). *αTUB2* and *ACT* were used because of their structural function and relatively low constitutive expression in barley (Schroder et al. 2001, T. Gjetting, *unpublished data*).

In order to avoid vector contamination, specific primers were designed from the 3′ part of the cDNA of the selected genes and were used in PCR with whole-leaf cDNA from Pallas plants or plasmid clones as template (sequences and primers are available upon request). The PCR products were excised from agarose gel, were purified using a Geneclean II kit (Qbiogene Inc., Carlsbad, CA, U.S.A.), and were immobilized on Hybond N^+ membranes, using a 96-well dot-blotting apparatus (Bio-Rad, Hercules, CA, U.S.A.). Identical membranes were produced with the 20-cell cDNA probes (30 ng per spot), using an electronic 8-channel multidispenser (Biohit PLC, Helsinki, Finland). The DNA was denatured and UV-cross-linked. Radioactive hybridization samples were prepared by random labeling with [α-^{32}P]-dCTP of 2 μl of the amplified single-cell cDNA pool, using standard techniques (Feinberg and Vogelstein 1983). Prior to labeling, excess nucleotides and primers were removed by a PCR product-purification kit (Qiagen Inc., Valencia, CA, U.S.A.). Labeling of first-strand cDNA synthesis from samples of total RNA (5 μg) was performed with a $(dT)_{20}$ primer (0.5 pmol) and a nucleotide mix containing dATP, dTTP, dGTP (0.5 mM each), dCTP (2 μM), and [α-^{32}P]-dCTP (0.67 μM, 30 μCi), according to Thordal-Christensen and associates (1992). Radioactive samples were purified using the Qiaquick Nucleotide Removal Kit (Qiagen, Inc.). Tube hybridization conditions were as described by Sambrook and associates (1988) but with dextran sulphate (5%). The membranes were washed at 65°C twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS for 10 min, twice in 1× SSC, 0.1% SDS for 10 min, then once at 68°C in 0.1× SSC, 0.1 % SDS for 10 min. The radioactive signals on membranes were analyzed by phosphorimager (Molecular Imager FX, Bio-Rad) and were quantified using Quantity One (Bio-Rad).

After background subtraction, intensity values representing transcript expression for each gene in each of the different cell classes (resistant, haustorium-containing, and controls) were obtained for the three independent, replicate experiments. Statistical analyses of variance of these data revealed that values for *UBC* showed little variation between cell classes or experiments and less variation than any other gene. Therefore, intensity values for all other genes in each cell class from all experiments were standardized against *UBC* (by dividing by the corresponding *UBC* value).

The cDNA pools used to test effects of 25, 35, or 45 PCR cycles were used as probes on dot-blot filters that included three of the housekeeping genes, eight genes known to be upregulated in response to *B. graminis* attack, and eight barley ESTs. Filters and radioactive samples were prepared as described above. However, for the 25-, 35-, and 45-cycle PCR procedures, labeling was with 5, 2, and 1 μl of the amplified single-cell cDNA, respectively.

ACKNOWLEDGMENTS

This work was supported in part by grant number 9901253 from the Danish Program Committee of Biotechnology, the Danish Research Council and by grant number 93S-953-00088 from the Danish Directorate for Food, Fisheries and Agri Business, Ministry of Food, Agriculture and Fisheries.

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